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VHDL, a larval storage protein from the corn earworm, *Helicoverpa zea*, is a member of the vitellogenin gene family ☆

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Abstract

The hemolymph of last instar larvae of the corn earworm, *Helicoverpa zea* contains a blue very high-density lipoprotein (VHDL) that is selectively taken up into fat body prior to pupation. Its amino-terminal sequence was determined by Edman degradation, and used to design a degenerate primer for PCR amplification. With 5′ and 3′ RACE techniques, the entire cDNA coding for VHDL was amplified and sequenced. Conceptual translation reveals a 173 kDa protein that contains a 15 amino acid signal sequence immediately before the experimentally determined N-terminus of the mature protein. The protein contains a typical lipoprotein N-terminal domain, and shows high sequence similarity to vitellogenins from Lepidoptera and other insect species. VHDL mRNA was not detectable in adult *H. zea*, and antibodies raised against VHDL did not react with adult hemolymph or yolk proteins. Therefore VHDL, although a member of the vitellogenin gene family, seems to be distinct from the vitellogenin expressed in adult females.
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1. Introduction

Holometabolous insects undergo major changes during their postnatal development. Metamorphosis, that is the transition from the larval to the adult stadium, is centered around the pupal stadium, during which many larval components are broken down, and adult proteins, tissues and structures are formed (Sehnal, 1985). Yet, pupae are physically separated from their surroundings and consequently unable to take in nutrients that can serve as precursors for the vast biosynthetic activities that must occur in this stadium. Instead, actively feeding larvae produce special storage proteins, which towards the end of the larval stadium are taken up from the hemolymph and stored in the fat body as dense protein granules (Levenbook, 1985). These granules are digested later in the pupal stadium to give rise to the amino acid precursors needed for the synthesis of adult proteins.

Most insect storage proteins are large hexameric proteins with subunits of approximately 80 kDa. These "hexamerins" include proteins with different characteristics, such as arylphorin (rich in aromatic amino acids), methionine-rich proteins, or proteins colored due to non-covalently bound riboflavin or biliverdin (Burmester and Scheller, 1999; Haunerland, 1996). All hexamerins are evolutionary related and belong to an ancient gene family that also includes the crustacean oxygen carrier hemocyanin (Beintema et al., 1994). While members of this gene family fulfill various and diverse functions, they are often highly expressed and accumulate at very high concentrations in the hemocoel. Their conserved structure may also contribute to the efficient uptake into fat body and subsequent storage in crystalline protein granules.

Some lepidopteran insects have been shown to employ not only hexameric storage proteins, but also a storage protein with characteristics distinct from hexamerins. A very high-density lipoprotein (VHDL) composed of subunits of

Abbreviations: NUP, nested universal primer; NTR, reverse N-terminal primer; UPM, universal primer mix; RACE, rapid amplification of cDNA ends; VHDL, very high-density lipoprotein

☆The sequence has been deposited in Genbank (Accession no. EF383143).

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~150 kDa was first identified in the hemolymph of last instar larvae of the corn earworm *Helicoverpa zea* (Haunerland and Bowers, 1986a). VHDL, colored brilliantly blue due to non-covalently bound biliverdin, contains approximately 10% lipid and has a density of 1.26 g/ml. Just like arylphorin, which is also present in *H. zea* (Haunerland and Bowers, 1986b), VHDL is synthesized in the fat body at the beginning of the last larval instar, and released in the hemolymph, where it accumulates at concentrations up to 10 mg/ml (Haunerland et al., 1990). Shortly before pupation, both storage proteins are taken up by endocytosis into fat body, apparently mediated by a common receptor (Wang and Haunerland, 1994), where they subsequently form dense protein granules that are partially broken down while the pharate adult develops.

Subsequently, similar blue VHDLs have been identified in other noctuid species, namely *Heliothis virescens* (Greenstone et al., 1991), *Spodoptera litura* (Yoshiga and Tojo, 1995), and *Trichoplusia ni* (Jones et al., 1988). Hymenopteran insect species (*Polistes metricus* and other Vespidae, Hunt et al., 2003; *Apis mellifera*, Shipman et al., 1987) have also been shown to possess a very high density, larval specific serum protein of a similar molecular weight, however without the colored chromophore.

While clearly different from hexamerins, the primary structures of VHDLs remain unknown. The present study was undertaken to obtain the complete sequence for VHDL from *H. zea*, and determine its relationship to other insect proteins.

2. Materials and methods

2.1. Insect rearing

Helicoverpa zea larvae, were obtained from the Department of Entomology, University of North Carolina, and reared on a 16 h light/8 h dark cycle at 26 °C. Larvae were housed individually in plastic containers containing an artificial diet.

2.2. Protein isolation and N-terminal sequence analysis

VHDL and the yolk protein vitellin were purified by differential density gradient centrifugation as previously described (Haunerland et al., 1987). N-terminal sequence analysis by Edman degradation was carried out at the Department of Biochemistry, University of Arizona, and Nucleic Acid Protein Service Unit, University of British Columbia.

2.3. Western blotting

Proteins were separated on a 10% T, 5% C SDS polyacrylamide gel and electrophoretically transferred onto PVDF membrane, as previously described (Persaud and Haunerland, 2004). Western blots were carried out with the ECL system (GE Health Care Bio-Sciences, Baie d'Urfé, PQ). The concentrations of primary antibody (rabbit anti-VHDL, Haunerland and Bowers, 1986a) and secondary antibodies (goat anti-rabbit IgG-HRP conjugate) were 1:10,000 and 1:3000, respectively.

2.4. Extraction of RNA from H. zea fat body

Larvae were dissected within 48 h after the final larval ecdysis, and fat body was placed immediately into RNAlater (Ambion, Austin, TX). If not used immediately for RNA extraction, the samples were stored at −20 °C.

Fat body from four larvae (approximately 70 mg) preserved in RNAlater was blotted dry with sterile filter paper. Extraction of total RNA from *H. zea* fat body was done using the Aurum Total RNA Fatty and Fibrous Tissue Kit (BioRad, Missisauga, Ont.), following the manufacturer's protocol with modifications to increase RNA recovery. The use of DNase I was omitted. Fat body was placed into 1 ml of chilled PUREzol (BioRad, Missisauga, Ont.) and homogenized for 60 s with a glass homogenizer. The lysate was left at room temperature for 5 min to allow the complete dissociation of nucleic acid/protein complexes. Chloroform (200 µl) was added and the sample was spun at 13,000 rpm for 15 min at 4 °C. The top, aqueous layer was loaded onto the spin columns, washed with the supplied high and low stringency wash solutions, and RNA was eluted with DEPC-treated water to yield a final volume of 50 µl.

RNA quality was assessed by electrophoresis in a 0.5% agarose gel, and by RT-PCR with primers specific for beta-actin (forward primer 5′-CCC ATC GAG CAC GGT ATC ATC AC-3′, reverse primer 5′-GCGTGGGGCAGAGCG-T-3′, amplicon size 314 bp). Using Ready-To-Go™ RT-PCR beads (GE Health Care Bio-Sciences, Baie d'Urfé, PQ), reverse transcription was carried out for 20 min at 42 °C, followed by 5 min at 95 °C and 35 cycles of 20 s at 95 °C, 20 s at 57 °C, and 30 s at 72 °C.

2.5. 3′ and 5′ RACE library construction

RACE libraries were constructed using the SMART™ RACE cDNA Amplification kit (BD Biosciences, Missisauga, Ont.). All reactions were carried out in 0.5 ml microcentrifuge tubes. The following reagents were added for first-strand synthesis to create a 5′RACE cDNA library: 1 µl total RNA, 1 µl 5′-CDS (12 µM) primer (5′-$(T)_{25}$VN-3′), 1 µl BD SMART II A Oligo (5′-AAG CAG TGG TAT CAA CGC AGA GTA CGC GGG-3′) oligonucleotide (12 µM) and 2 µl sterile H_2O. For the creation of a 3′RACE cDNA library, the following reagents were added: 1 µl total RNA, 1 µl 3′-CDS (12 µM) primer (5′-AAG CAG TGG TAT CAA CGC AGA CTA $C(T)_{30}$VN-3′) and 3 µl sterile H_2O. Reactions were incubated at 70 °C for 2 min followed by cooling on ice for 2 min. The following was then added to both reactions in the following order: 2 µl 5X first-strand buffer, 1 µl DTT (20 mM), 1 µl dNTP mix (10 mM) and 1 µl BD

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